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Multicentre study of the prevalence of toxigenic *Clostridium difficile* in Korea: results of a retrospective study 2000–2005

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The prevalence of toxigenic *Clostridium difficile* in Korea has been reported to be approximately 60–80 %. Although the prevalence of the *tcdA*$^-$*tcdB*$^+$ *C. difficile* strain was less then 5 % prior to the year 2000, it has become an emerging nosocomial pathogen in Korea. Therefore, we have attempted to determine the multicentre nationwide prevalence of *tcdA*$^+$*tcdB*$^+$ and *tcdA*$^-$*tcdB*$^+$ *C. difficile* for epidemiological purposes. *C. difficile* strains (n=724, 30 from 2000, 80 from 2001, 74 from 2002, 76 from 2003, 179 from 2004, 285 from 2005) were obtained retrospectively from January 2000 to December 2005 from in-patients at 6 hospitals, all of whom were suspected of having *C. difficile*-associated disease (CDAD), colitis or pseudomembranous colitis. The numbers of participating hospitals varied yearly (1 in 2000, 2 in 2001–2003, 3 in 2004, 5 in 2005). The hospitals were located in Seoul (n=4), Kyunggi Province (n=1) and Busan (n=1), Korea. PCR assays for *tcdA* and *tcdB* genes were conducted using 724 unduplicated *C. difficile* isolates. The mean prevalence of *tcdA*$^+$*tcdB*$^+$ and *tcdA*$^-$*tcdB*$^+$ *C. difficile* strains over the 6 years was 51.8 % (38.4–59.3 %) and 25.8 %(10–56.0 %), respectively. The mean prevalence of *tcdA*$^-$*tcdB*$^+$ *C. difficile* strains was less than 7 % until 2002, but began to increase in 2003 (13.2 %) and achieved a peak in 2004 (50.3 %). In 2005, the mean prevalence of *tcdA*$^+$*tcdB*$^+$ and *tcdA*$^-$*tcdB*$^+$ *C. difficile* strains was 47.7 % (30.9–60.3 %) and 27.0 % (17.6–54.8 %), respectively. This nationwide epidemiological study showed that *tcdA*$^-$*tcdB*$^+$ *C. difficile* strains have already spread extensively throughout Korea, and our results provide basic data regarding the controversies currently surrounding the toxigenicity of *tcdA*$^-$*tcdB*$^+$ *C. difficile*. The use of enzyme immunoassays capable of detecting both TcdA and TcdB is strongly recommended for the diagnosis of CDAD in microbiology laboratories, in order to control the spread of the *tcdA*$^-$*tcdB*$^+$ strains of *C. difficile*.

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INTRODUCTION

Clostridium difficile is one of the most common nosocomial pathogens and is responsible for *C. difficile*-associated disease (CDAD) and pseudomembranous colitis (PMC). *C. difficile* can be either toxigenic or non-toxigenic. Toxigenic *C. difficile* strains generally produce an enterotoxin (TcdA) and a cytotoxin (TcdB). These toxins are encoded by two genes, *tcdA* and *tcdB*, which have been mapped to a 19.6 kb chromosomal pathogenicity locus (PaLoc) (Rupnik *et al.*, 1998). Besides both of these genes, three additional

Abbreviations: CDAD, *Clostridium difficile*-associated disease; EIA, enzyme immunoassay; PaLoc, pathogenicity locus; PMC, pseudomembranous colitis.

regulatory genes (*tcdC, tcdD* and *tcdE*) are located within the PaLoc (Braun *et al.*, 1996; Hammond & Johnson, 1995; Cohen *et al.*, 2000). However, a number of different genetic variants of *C. difficile* have been reported with increasing frequency worldwide. *C. difficile* strains with various genetic modifications within the PaLoc have been studied in the past, and 28 different toxinotypes have been identified (Rupnik *et al.*, 1998). These include variants of *C. difficile* that harbour deletions, insertions or polymorphic restriction sites in one or more of the genes within the PaLoc, but still generate functional TcdA and TcdB toxins ($TcdA^+TcdB^+$ strains). However, strains of toxinotypes VIII, X, XVI and XVII generate a functional TcdB but no TcdA ($TcdA^-TcdB^+$ strains), whereas strains grouped into toxinotype XI harbour only the 3′ portion of *tcdA* and generate neither TcdA nor TcdB ($TcdA^-TcdB^-$strains) (Von Eichel-Streiber *et al.*, 1999; Rupnik *et al.*, 1997; Rupnik, 2001). Until recently, it was believed that all *C. difficile* strains responsible for diseases generated both TcdA and TcdB, which functioned synergistically. However, a great many recent studies have shown that $TcdA^-TcdB^+$ strains are involved in a wide spectrum of CDAD ranging from colonization, to uncomplicated diarrhoea to PMC (Brazier *et al.*, 1999; Al-Barrak *et al.*, 1999; Alfa *et al.*, 2000; Limaye *et al.*, 2000; Johnson *et al.*, 2001; Kuijper *et al.*, 2001; Pituch *et al.*, 2001).

The prevalence rates of these $TcdA^-TcdB^+$ strains were reported as ranging from 0.2 to 56 % in different studies from the USA, Europe and Asia (Kato *et al.*, 1998; Lyerly *et al.*, 1998; Pituch *et al.*, 2001; Barbut *et al.*, 2002; Samra *et al.*, 2002; Rupnik *et al.*, 2003; Geric *et al.*, 2004). However, as the $TcdA^-TcdB^+$ *C. difficile* prevalence rate has been reported to be as high as 39 % in one Japanese study (Komatsu *et al.*, 2003) and a highly prevalent $TcdA^-TcdB^+$ *C. difficile* strain was detected in a tertiary hospital in Korea, this variant strain has become recognized as an emerging pathogen in Korea (Shin & Kuak, 2006; Shin *et al.*, 2008). Therefore, it is important to determine at what time the $TcdA^-TcdB^+$ *C. difficile* strains became prevalent and when they really began to be widely distributed throughout Korea. In this study, we have investigated the nationwide prevalence of $TcdA^+TcdB^+$ and $TcdA^-TcdB^+$ *C. difficile* strains in Korea from 2000 to 2005 for epidemiological purposes.

METHODS

Specimens. A total of 724 strains of *C. difficile* (30 from 2000, 80 from 2001, 74 from 2002, 76 from 2003, 179 from 2004, 285 from 2006) were obtained between January 2000 and December 2005 from in-patients at 6 hospitals suspected of harbouring CDAD, colitis or PMC. The numbers of participating hospitals varied yearly (1 in 2000, 2 in 2001–2003, 3 in 2004, 5 in 2005). The hospitals were located in Seoul (n=4), Kyunggi Province (n=1) and Busan (n=1), Korea, and the mean number of beds was 920, with a range of 400–2000 (Fig. 1).

***C. difficile* cultures.** Stool samples collected from patients suspected to be infected with *C. difficile* were inoculated into anaerobically reduced cycloserine–cefoxitin–fructose agar at 37 °C under anaerobic conditions for 48–72 h in each participating hospital. The identification of *C. difficile* was conducted in each participating laboratory in accordance with local techniques, which included analysis of Gram stain, spore stain, characteristic odour and typical morphological features, and/or enzyme immunoassays (EIAs) to detect TcdA. Isolates sent to the central coordinating laboratory were then subcultured, and identification was confirmed via a biochemical assay using an ANA identification test kit (bioMérieux).

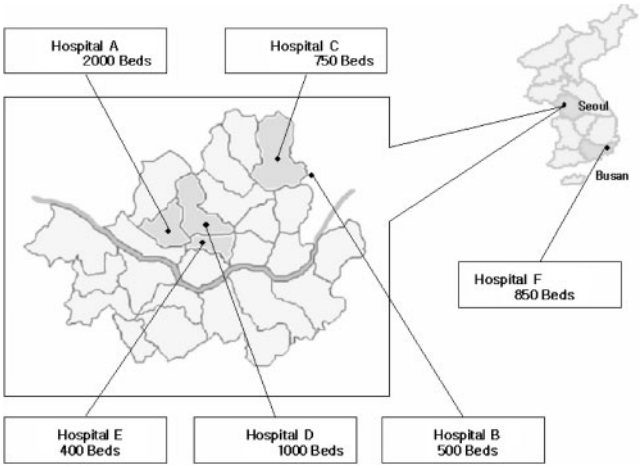

Fig. 1. The location and number of beds for each of the six participating hospitals.

PCR assay for *tcdA* and *tcdB*. Genes that encode the large clostridial toxins A and B (*tcdA* and *tcdB*) were detected by PCR in accordance with the methods described by Kato *et al.* (1998), with some modifications, on 724 strains of *C. difficile*. Template DNA was prepared by suspending 20 colonies in a 5 % (w/v) solution of Chelex-100 (Bio-Rad), boiling for 12 min, then centrifuging for 5 min at 12 000 ***g***. The total volume of the PCR reaction was 100 µl, and it contained ~30 ng bacterial DNA preparation, 0.15 µg each primer, the four dNTPs (200 µM each), 10 mM Tris/HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl and 2.5 U *Taq* polymerase. *tcdA* and *tcdB* were amplified using the following primers: NK9 (5′-CCACCA GCTGCAGCCATA-3′) and NK11 (5′-TGATGCTAATAATGAA TCTAAAATGGTAAC-3′), which were derived from the repeating sequence of *tcdA*; and primers NK104 (5′-GTGTAGCAATGAAA GTCCAAGTTTACGC-3′) and NK105 (5′-CACTTAGCTCTTT GATTGCTGCACCT-3′), which were derived from the non-repeating sequence of *tcdB*, respectively. The ATCC 43596 strain (serogroup C) was utilized as the $tcdA^+tcdB^+$ control and ATCC 43598 strain (serogroup F) was used as the $tcdA^-tcdB^+$ variant control. PCR amplification using NK9/NK11 was conducted in a thermal cycler (Perkin-Elmer) with 40 cycles of 95 °C for 15 s, 62 °C for 120 s and 72 °C for 40 s. For the primer pairs NK104/NK105 as the following thermal profile was used: 40 cycles at 95 °C for 20 s, 62 °C for 60 s and 74 °C for 40 s. At the conclusion of these PCR cycles, the tubes were incubated for 5 min at 74 °C. Following completion of the PCRs, 10 µl amplified product was electrophoresed in 2 % agarose gel and the bands were visualized by UV transillumination.

Strains in which the *tcdA* gene was intact yielded 1200 bp PCR products, and $tcdA^-tcdB^+$ variant strains, yielded 700 or 500 bp PCR products. In *C. difficile* strains with an intact $tcdB^+$ gene, the PCR product for *tcdB* was 204 bp.

RESULTS

The total number of *C. difficile* isolates studied was 724. As we conducted the PCR assay for *tcdA* and *tcdB* retrospectively, the number of strains available was only 30 in 2000, but the number was increased to 285 in 2005. The prevalences of $tcdA^+tcdB^+$ and $tcdA^-tcdB^+$ *C. difficile* strains were 93.3 and 6.7 % in 2000. No $tcdA^-tcdB^-$ *C. difficile* strain was detected in that year. However, the prevalence of the $tcdA^+tcdB^+$ strain showed a tendency toward decrease from 2001 (73.8 %) and fell to its lowest level in 2004, as low as 35.2 %. In 2005, the mean prevalence of $tcdA^+tcdB^+$ *C. difficile* was 47.7 %, ranging from 30.9 to 60.3 %. The prevalence of the $tcdA^-tcdB^+$ strain began to increase, up to 13.2 % in 2003, and finally up to 50.3 % in 2004. In 2005, the mean prevalence of the $tcdA^-tcdB^+$ strains was 27.0 %, ranging from 17.6 to 54.8 %. The $tcdA^-tcdB^+$ variant strains appeared to become endemic nationwide beginning in 2005. The mean prevalence of $tcdA^-tcdB^-$ *C. difficile* strains was approximately 22.4 % for 6 years, ranging from 0 % (2000) to 32.9 % (2003).

Although the mean prevalences of the $tcdA^+tcdB^+$ and $tcdA^-tcdB^+$ *C. difficile* strains over the 6 years were 51.8 % (range 33.3–69.9 %) and 25.8 % (range 10–56 %), the distributions of $tcdA^+tcdB^+$ and $tcdA^-tcdB^+$ *C. difficile* strains differed from hospital to hospital and from year to year (Fig. 2). In 2000, the *C. difficile* strains were collected only from one hospital (hospital A) and the prevalence of the variant strain in that hospital was 6.7 %. In 2001, isolates of *C. difficile* were acquired from two hospitals (hospitals A and B). However, the variant strains were not observed in the participating hospital in 2000, although the mean prevalence of the variant strain was 5 %. In 2002, the rate of prevalence of the variant strain was 4.2 % (hospital A) and 6.0 % (hospital B), respectively. In 2003, the prevalence of the variant strains began to increase up to 16.7 % in one hospital, whereas it remained 7.1 % in the other participating hospital. In 2004, another two hospitals newly participated in the surveillance (hospitals C and D), and one hospital (hospital B) did not participate in the follow-up. One of them (hospital D) reported that the prevalence of $tcdA^-tcdB^+$ *C. difficile* remained less than 7 % (6.4 %), but the other hospital (hospital C) reported highly prevalent $tcdA^-tcdB^+$ *C. difficile* strains (71.8 %), suggesting an outbreak. The other continuing participating hospital (hospital A) reported an increased prevalence of $tcdA^-tcdB^+$ strains (36.4 %), which was also suggestive of outbreak.

In 2005, another two hospitals (hospitals E and F) joined the surveillance, and they already had highly prevalent $tcdA^-tcdB^+$ strains (25.6 and 54.8 %, respectively). Their $tcdA^+tcdB^+$ and $tcdA^-tcdB^-$ *C. difficile* rates were 53.9/20.5 % (hospital E) and 30.9 %/14.3 % (hospital F), respectively.

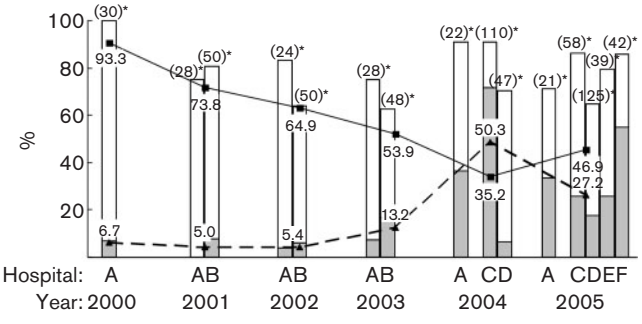

Fig. 2. The annual prevalence rate of $tcdA^+tcdB^+$, $tcdA^-tcdB^+$ and $tcdA^-tcdB^-$ *C. difficile* strains in each participating hospital from 2000 to 2005. White bars, prevalence rate (%) of $tcdA^+tcdB^+$ *C. difficile* strain in each hospital; grey bars, prevalence rate (%) of $tcdA^-tcdB^+$ *C. difficile* strains in each hospital; ■ mean prevalence rate (%) of $tcdA^+tcdB^+$ *C. difficile* strains in all participating hospitals; ▲ mean prevalence rate (%) of $tcdA^-tcdB^+$ *C. difficile* strains in all participating hospitals; *, number of strains tested for each year.

DISCUSSION

$TcdA^-TcdB^+$ *C. difficile* has been reported in several countries, with varying prevalence rates. As highly prevalent $tcdA^-$ variant strains were previously isolated from patients with CDAD in a tertiary hospital in Korea (Shin & Kuak, 2006; Shin *et al.*, 2008), further nationwide surveillance was required in order to sufficiently ascertain the prevalence and virulence of $tcdA^-tcdB^+$ strains of *C. difficile* in Korea. As published studies revealed no $tcdA^-tcdB^+$ strains among *C. difficile* isolates in Korea (Kato *et al.*, 1998; Rupnik *et al.*, 2003) or lower prevalence rates (4.3 %) of these strains (Chung *et al.*, 2002), we needed to determine at what time these *C. difficile* strains became prevalent and truly widely spread throughout Korea. This study is believed to be the first nationwide surveillance of *C. difficile* isolates from patients suspected of having CDAD in Korea.

We retrospectively obtained 724 *C. difficile* strains stored in each of 6 hospitals over 6 years, from 2000 to 2005. The mean prevalence of $tcdA^-tcdB^+$ strains was less than 10 % (range 5.5–6.7 %) until 2002, but it almost doubled (13.2 %) in 2003 and reached a maximum at 2004, with levels as high as 50.3 %. The mean prevalence of $tcdA^-tcdB^+$ strains in 2005 was 27.0 % (range 17.6–54.8 %), which was approximately four times higher than was reported in 2000.

The prevalence rates of $TcdA^-TcdB^+$ strains were reported to be 1.3–2 % in the USA (Lyerly *et al.*, 1998; Geric *et al.*, 2004) and 2.7 % in France (Barbut *et al.*, 2002). In the UK, it was estimated that approximately 3 % of toxigenic *C. difficile* are $TcdA^-TcdB^+$ strains (Brazier *et al.*, 1999). It was identified in 11 % of CDAD cases in Poland (Pituch *et al.*, 2001). A recent 2 month prospective study in Europe in 2005 showed that 24.3 % of *C. difficile* isolates were toxin

variants, and the prevalence rate of toxinotype VIII was 5 % overall among them (Barbut *et al.*, 2007). By way of contrast, they reported that PCR ribotype 017 was particularly predominant in Poland, Ireland, Greece and Sweden, and all PCR ribotype 017 isolates belonged to toxinotype VIII and were *tcdA*$^-$*tcdB*$^+$ strains. Other recent studies reported that *tcdA*$^-$*tcdB*$^+$ strains accounted for 56 % in Israel and 44 % in Ireland (Samra *et al.*, 2002; Drudy *et al.*, 2007).

As compared with these reports, the prevalence of *tcdA*$^-$*tcdB*$^+$ strains in Korea was less than or similar to that of the other countries until 2002−2003, but has increased since 2004. Our results revealed that outbreaks of *tcdA*$^-$*tcdB*$^+$ strains were experienced in two hospitals in 2004; infection with these strains remained highly prevalent in 2005, as compared with published reports in Korea (Shin & Kim,1992; Lee & Chung, 1993; Lee *et al.*, 1999; Kang *et al.*, 2000; Chung *et al.*, 2002). One hospital (hospital F), which was involved only in 2005, is located 450 km from other participating hospitals. However, the prevalence of *tcdA*$^-$*tcdB*$^+$ strains in that hospital had already reached a level of 56 %. The prevalence of variant strains in Japan was reported to be 12.5 % (Kato *et al.*, 1998). However, variant prevalence rates as high as 39 % during a 1 year period (December 1999–November 2000) have been reported in CDAD patients, although this was a report issued by a Japanese hospital (Komatsu *et al.*, 2003). These results suggested that *tcdA*$^-$*tcdB*$^+$ strains had spread throughout Far East Asia, including Korea and Japan, before 2000, and these *tcdA*$^-$*tcdB*$^+$ strains appeared to be endemic in many Korean hospitals since 2004.

We are not currently precisely certain as to why we had such highly prevalent *tcdA*$^-$*tcdB*$^+$ strains in Korea over such a brief duration of time. We surmised that one reason might be that patients are constantly on the move seeking better medical service, and *C. difficile* is a pathogen that is quite difficult to eradicate, because of its ability to sporulate (McFarland *et al.*, 1989). Another factor may be a failure of infection control due to the lack of awareness of the physicians involved. It appears likely that, at some point, there must have been a clinical sign of a *C. difficile* variant. However, we may have missed this because we did not possess adequate data regarding the nationwide prevalence of *C. difficile* variants prior to 2005, because culturing, *C. difficile* is a strenuous procedure in the majority of laboratories, and EIAs for TcdA only have been widely utilized for the diagnosis of *C. difficile* infection in Korea, as has been reported in other European countries (Barbut *et al.*, 2003). These commercial toxin A EIAs are not capable of detecting *tcdA*$^-$*tcdB*$^+$ variants, as variant strains of *C. difficile* that harbour deletions of 1.7–1.8 kb in *tcdA* yielded no detectable TcdA (Kato *et al.*, 1998; Von Eichel-Streiber *et al.*, 1999; Rupnik *et al.*, 1997). Therefore, no attention appeared to be paid previously to infection control, especially with regard to *tcdA*$^-$*tcdB*$^+$ *C. difficile* strains. Another factor to be considered is that the diagnostic algorithms of CDAD differ from hospital to hospital. Therefore, these outbreaks appear to be associated with delays in CDAD diagnosis in certain hospitals. All of these factors may result in a significant burden on the health-care system associated with CDAD (Riley *et al.*, 1995).

In conclusion, this nationwide epidemiological study showed that *tcdA*$^-$*tcdB*$^+$ *C. difficile* strains have already spread widely in Korea, and EIAs capable of detecting both TcdA and TcdB should be utilized in microbiology laboratories in order to control these strains of *C. difficile* in Korea.

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